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# CXCR4: A Potential Marker for Inflammatory Activity in Abdominal Aortic Aneurysm Wall

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**WHAT THIS PAPER ADDS**

This study demonstrates that the expression of the chemokine receptor CXCR4 and its ligand CXCL12 is significantly upregulated in human AAA compared with non-aneurysmal aortic tissue. CXCR4 is mainly co-localized in inflammatory cells, particularly in B and T lymphocytes as well as macrophages. Consequently, CXCR4 might be involved in inflammatory proteolytic processes in the pathogenesis of AAA.

**Objectives:** The aim of the study was to evaluate the potential role of chemokine receptor CXCR4 and its ligand CXCL12 in the pathogenesis of abdominal aortic aneurysm (AAA).

**Methods:** AAA tissue specimens were obtained from the anterior or lateral aneurysm sac of patients ($n = 32$, 26 males, 6 females; 66.8 $\pm$ 11.2 years, diameter 64.4 $\pm$ 17.0 mm), who underwent elective open surgical repair. Twelve non-aneurysmal aortic specimens from transplant donors served as controls. Expression analysis of CXCR4 and CXCL12 at mRNA and protein level was determined by quantitative reverse transcription–polymerase chain reaction (RT-PCR) and western blot. Immunohistochemical staining of corresponding histological sections for CD3 (T-cells), CD20 (B-cells), and CD68 (macrophages) was performed to determine the cellular localization of CXCR4 and CXCL12. Data were analyzed with SPSS 20.0 using Mann–Whitney U test and Spearman's rank correlation coefficient.

**Results:** Gene expression of *CXCR4* and *CXCL12* was 9.6 and 4.6 fold higher in AAA than in non-aneurysmal aorta ($p = .0004$ and $p < .0001$, respectively). Likewise, the protein level of CXCR4 was increased 3.2 fold in AAA wall compared with non-aneurysmal aortic tissue ($p < .0001$), although CXCL12 could not be detected. Immunohistochemical analysis revealed that CXCR4 was expressed in B and T lymphocytes and macrophages, and CXCL12 was observed only in plasma cells.

**Conclusions:** This study confirmed the over expression of CXCR4 in human AAA tissue. CXCR4 was detected both at the mRNA and the protein level and by immunohistochemistry, especially in inflammatory cells. In contrast, CXCL12 expression was observed only at the mRNA level, with the exception of plasma cells. The exact role of CXCR4/CXCL12 in AAA has to be further elucidated.



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**Keywords:** Abdominal aortic aneurysm, CXCR4, CXCL12, Gene expression, Inflammatory processes

## INTRODUCTION

Abdominal aortic aneurysm (AAA) is a common pathology of the elderly population,[1,2] which may lead to rupture causing severe internal bleeding and sudden death.[3–6] The pathological dilatation of the aorta is considered to be the result of chronic inflammation, upregulation of proteolytic pathways, oxidative stress, degradation of the extracellular matrix (ECM), and vascular smooth muscle cell (VSMC) apoptosis.[6–11]

Inflammatory response and activation of proteolytic enzymes are widely controlled by a plethora of various chemokines and their receptors.[12–17] Increased attention has been paid to one particular chemokine receptor CXCR4, specific for stromal derived factor-1 alpha (SDF-1$\alpha$ also known as CXCL12), involved in the activation and chemotactic attraction of lymphocytes at the site of inflammation. Moreover, CXCR4 and its ligand CXCL12 appear to play a key role in many immunological areas such as organogenesis, vascularization, and embryogenesis.[18–20] Additionally,

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CXCR4 has already been investigated as a key receptor in the crosstalk between tumor cells and their microenvironment[21,22] and between the immune and the nervous systems.[23,24] The exact role of CXCR4 in human AAA is however still unknown. Ocaña et al.[25] have shown that CXCR4 was highly expressed in infiltrating cells, especially T and B lymphocytes, isolated from human AAA. Furthermore, the level of CXCL12 was increased in the adventitia of human AAA wall, presuming an important role of CXCR4/CXCL12 in the development of AAA.[26]

The aim of this study was to analyze the expression of CXCR4 and its ligand CXCL12 in human AAA tissue samples and compare the results with that of the non-aneurysmal aorta. Furthermore, the aim was to identify the cellular localization of CXCR4 and CXCL12 within the aortic wall.

## METHODS

### *Patients and tissue samples*

All AAA tissue samples ($n = 32$) were obtained, based on computed tomography (CT) data, from the anterior or lateral AAA sac at the maximum dilatation area of the AAA during elective open surgical repair. The AAA tissue samples were randomly excised from an anterior or lateral aneurysm sac depending upon the nature of the aneurysm, and on still being able to fully cover the aortic prosthesis. The average age of the study patients (26 males, 6 females) was 66.8 $\pm$ 11.2 years. The maximum AAA diameter derived from pre-operative CT-scans was 64.4 $\pm$ 17.0 mm. The demographic data of the study patients, including age, sex, smoking history, comorbidities such as coronary heart disease (CHD), hypertension, diabetes mellitus (DM), hyperlipidemia, medications (aspirin, beta blocker, statins, ACE (angiotensin converting enzyme) inhibitors, diuretics) obtained from the patient's medical report, are summarized in Table 1. Moreover, the glomerular filtration rate (GFR) was estimated according to clinical guidelines for the diagnosis, prevention, and treatment of chronic kidney disease (CKD).[27] Study individuals with eGFR $<$ 60 mL/min were considered as CKD patients. Non-aneurysmal aortic specimens from peri-renal abdominal aorta ($n = 12$, 10 males, 2 females; 45 $\pm$ 17 years), obtained from human organ donors during kidney transplantation at the Department of Transplant Surgery, Klinikum rechts der Isar, were used as controls. The aortic tissue samples from AAA patients and from the healthy donors were obtained from all three vessel wall layers: adventitia, media, and intima. Following excision, the tissue specimens were separated into two parts. One part was fixed in formalin and embedded in paraffin (FFPE), the other part was immediately frozen in liquid nitrogen. Fresh frozen samples were used for protein extraction and western blot analyses; quantitative real time reverse transcriptase—polymerase chain reaction (RT-PCR), histological, and immunohistochemical analyses were performed using FFPE samples.

Informed written consent was obtained from all patients. The study was performed with the approval of the ethics committee of the university hospital Klinikum rechts der Isar, Technische Universitaet Muenchen, Germany.

**Table 1.** Patient demographic data.

| | |
|---|---|
| Number of patients | 32 (26 males, 6 females) |
| Age (years) (median/range) | 68.5/40—87 |
| Max AAA diameter (mm) (median/range) | 59.5/40—90 |
| Number of patients with | *n*/% |
| Chronic kidney disease[a] | 12/37.5 |
| Hypertension[b] | 26/81.3 |
| Diabetes mellitus | 1/3.1 |
| Coronary heart disease[c] | 6/18.8 |
| Positive smoking status | 8/25 |
| Medication | *n*/% |
| ASA | 23/71.9 |
| Beta blocker | 20/62.5 |
| Statins | 18/56.3 |
| ACE inhibitors | 13/40.6 |
| Diuretics | 13/40.6 |

ACE = angiotensin converting enzyme ASA = aspirin.
[a] Estimated glomerular filtration rate $<$60 mL/min.
[b] Systolic/diastolic pressure exceeding 140/90 mmHg; six patients had normal blood pressure, three were without any treatment regarding antihypertensives, and the other three were treated with ACE inhibitors.
[c] All cardiac disorders including stable angina, unstable angina and myocardial infarction.

### *Chemokine mRNA determination by quantitative real time RT-PCR*

Quantitative real time RT-PCR was performed using FFPE tissue samples. Total RNA was isolated using High Pure RNA Paraffin Kit (Roche, Mannheim, Germany) and transcribed into complementary DNA with cDNA Synthesis Kit Revert Aid (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. SYBR Green fluorescence dye (peqLab, Erlangen, Germany) and SYBR Green Cycler StepOnePlus (Life Technologies, Darmstadt, Germany) were used to quantify PCR results. PCR conditions were as follows: initial PCR activation step for 5 minutes at 95 °C, followed by 45 thermal cycles of denaturation for 10 seconds at 95 °C, annealing temperature for 30 seconds at 60 °C, extension for 10 seconds at 72 °C, and an additional 15 seconds at 77 °C to eliminate primer dimers. The mRNA levels for each target gene were standardized to the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following commercial available primers were used: QuantiTect Primer Assay (Qiagen, Hilden, Germany): *GAPDH* (Hs_GAPDH_1_SG, NM_001256799, 95 bp) *CXCR4 (*Hs_CXCR4_1_SG, NM_001008540, 106 bp), *CXCL12* (*SDF-1α*) (Hs_CXCL12_1_SG, NM_000609, 70 bp) *CD3* (cluster of differentiation 3; T-cell marker) (Hs_CD3D_1_SG, NM_000732, 72 bp), *MSR1* (macrophage scavenger receptor 1) (Hs_MSR1_1_SG, NM_002445, 91 bp), and *CD45* (cluster of differentiation 45; leucocytes marker) (Hs_PTPCR_5_SG, NM_002838, 159 bp)

### Protein extraction and western blot analysis

Tissue samples were homogenized in liquid nitrogen using a mortar and pestle and suspended in RIPA buffer (50 mm Tris-HCl [pH 7.5], 150 mm sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate), supplied with freshly prepared protease inhibitor mix (Roche, Mannheim, Germany) for 30 minutes on ice. Samples were centrifuged at 14,000 $\times$ *g* for 30 minutes at 4 °C and the supernatant containing protein extract was used for further analysis. The protein concentration was determined using the BCA Protein Assay Kit (Pierce, Thermo Scientific, Rockford, IL, USA). Equal amounts of protein from each sample (50 µg) were fractioned by sodium dodecyl sulfate—polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 hour with Tris-buffered saline (TBS) containing 5% (w/v) milk and 0.1% Tween and incubated with the primary antibody overnight at 4 °C. The blots were washed with TBS containing Tween, incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (R&D Systems Inc., Minneapolis, MN, USA) for another hour at room temperature. The specific proteins were detected by the chemiluminescence reaction (Thermo Scientific, Rockford, IL, USA). Protein quantification was performed by comparing the band intensity of each sample with the intensity of the corresponding GAPDH band. The following primary antibodies were used: anti-CXCR4 polyclonal antibody (ab2074, Abcam Inc., Cambridge, UK; dilution 1:1,000), anti-SDF-1α (ab9797, Abcam Inc., Cambridge, UK; dilution 1:1,000), and GAPDH (ab8245, Abcam Inc.; dilution 1:10,000). The following secondary antibodies were used: goat anti-rabbit IgG (ab97200, Abcam Inc.; dilution 1:5,000), goat anti-mouse IgG (130043, KPL, Gaithersburg, USA; dilution 1:10,000). As a positive control for western blot analyses, active human SDF1 beta full length protein (abcam) was used.

### Histological and immunohistochemical staining

Histological and immunohistochemical staining analyses were performed as follows: cross sections (3 µm thick) were cut from AAA and control tissue blocks, mounted on slides treated with 0.1% poly-L-lysine solution (Sigma-Aldrich, St. Louis, MO, USA) for better adhesion, and dried for 1—2 hours at 56 °C before staining. Hematoxylin and eosin (HE) and Elastica van Gieson (EvG) staining were performed to characterize the morphology of the aortic wall. For immunohistochemical staining, the sections were deparaffinized, rehydrated, placed in 10 mM citrate buffer (pH 6.0), and treated in a pressure cooker for 7 minutes. For CXCR4 and CXCL12, a target retrieval buffer with pH 9.0 (S2367, Dako REALTM, Inc) was used and sections were treated for 20 minutes in a microwave, pre-incubated with 0.3% hydrogen peroxide ($H_2O_2$) for 15 minutes at room temperature to block endogenous peroxidase activity. Subsequently, the sections were pre-treated with 1% bovine serum albumin in background reducing diluents (S2022, Dako REAL, Inc) and then incubated with the appropriate antibodies: anti-CXCR4 antibody (ab124824, Abcam Inc.; dilution 1:300), anti-SDF-1α (CXCL12) antibody (ab9797, Abcam Inc.; dilution 1:500/1,000), T lymphocytes (anti-CD3, MRQ-39, Cell Marque; dilution 1:500), B lymphocytes (anti-CD20, M0755, clone L26, Dako; dilution 1:500), macrophages/monocytes (anti-CD68, M0814, Dako; dilution 1:2,000), and plasma cells (anti-VS38c, M7077, Dako, dilution 1:1,000). Thereafter, the sections were incubated with biotinylated secondary antibody for 25 minutes, followed by peroxidase conjugated streptavidin for another 25 minutes. Finally the sections were incubated in 3,3'-diaminobenzidine (DAB) chromogen for 3—5 minutes, counterstained with Mayer's hematoxylin, and mounted. Exclusion of the primary antibody during immunostaining was used as a negative control, while tonsil sections served as a positive control.

### Statistical evaluation

All statistical analyses were performed using SPSS for Windows version 20.0 (SPSS Inc., Chicago, IL, USA). To compare values of continuous variables, a non-parametric Mann—Whitney U test was used. Correlations between continuous variables were quantified using Spearman's rank correlation coefficient. All statistical tests were two sided. A $p$ value $< .05$ was considered significant.

## RESULTS

### Expression of CXCR4/CXCL12 at mRNA level

Using quantitative real time RT-PCR, the mRNA levels of the chemokine receptor *CXCR4* and its ligand *CXCL12*, as well as the inflammatory markers for T and B lymphocytes and macrophages were detected in the AAA wall excised from the anterior or lateral sac at the maximum dilatation site as well as in the control tissue samples (Fig. 1). Following standardization to the expression of *GAPDH*, AAA samples showed significantly increased mRNA expression levels for *CXCR4* compared with control aorta by 9.6 times ($p = .0004$). The expression of *CXCL12* was increased 4.6 fold ($p < .0001$), *CD45* 6.3 fold ($p < .0001$), *CD3* 10.3 fold ($p = .014$), and *MSR1* 16.6 fold ($p < .001$) in comparison with the control subjects.

Furthermore, because the study groups (AAA vs. control aorta) were not age matched, correlation analysis between age and the expression of *CXCR4* and *CXCL12* in the AAA patients and in the control group was performed, calculating the Spearman rank correlation coefficient. No significant relationships were observed (AAA group: $\rho = -0.38$; $p = .849$ for CXCR4 and $\rho = .104$; $p = .570$ for CXCL12; control group: $\rho = .447$; $p = .168$ for CXCR4, and $\rho = .451$; $p = .190$ for CXCL12).

### Relationship between the gene expression of CXCR4, CXCL12, and selected inflammatory markers

As the chemokine receptor *CXCR4* is known to be involved in lymphocyte activation, differentiation, and trafficking at the site of inflammation, it was also determined whether

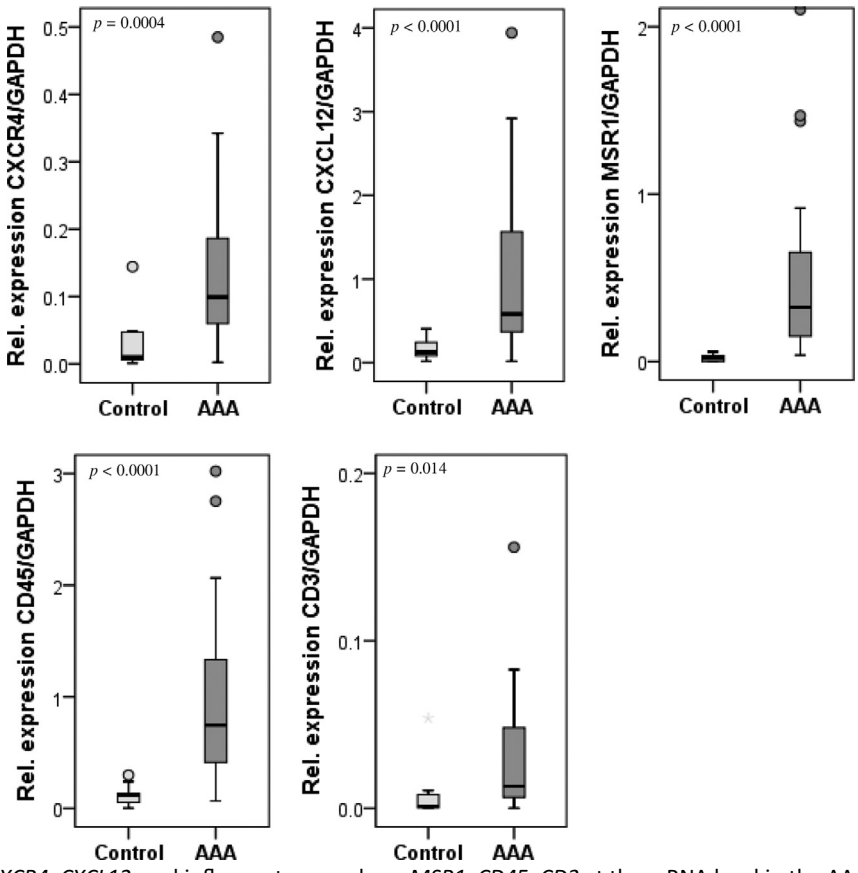


**Figure 1.** Expression of *CXCR4*, *CXCL12*, and inflammatory markers: *MSR1*, *CD45*, *CD3* at the mRNA level in the AAA tissue samples ($n = 32$) compared with healthy control tissues ($n = 12$) analyzed by quantitative real time polymerase chain reaction and SYBR green fluorescence dye; the expression levels were standardized to GAPDH. The Mann–Whitney U test was used. AAA = abdominal aortic aneurysm; CD, cluster of differentiation; *MSR1*, macrophages scavenger receptor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

any relationship existed between *CXCR4*, *CXCL12*, and inflammatory cells in the AAA wall (Table 2). Positive correlations were observed between the expression of *CXCR4* and the expression of *CXCL12*, *MSR1*, *CD45*, and *CD3* ($p < .001$, $p = .002$, $p < .001$ and $p = .002$, respectively). Furthermore, *CXCL12* was significantly correlated with the inflammatory markers *MSR1*, *CD45*, and *CD3* as well ($p < .001$, $p < .001$, and $p = .001$, respectively).

### *Expression of CXCR4/CXCL12 at protein level*

Following expression analysis at the mRNA level, the expression of CXCR4 and CXCL12 in the AAA tissue samples was determined at the protein level using western blot analysis (Fig. 2). The expression of CXCR4 was found in AAA and in the control group at the expected protein size (43 kDa). In contrast, the expression of CXCL12 (11 kDa) at protein level could not be detected. An example of the protein bands is shown in Fig. 2. The protein level of CXCR4 in AAA tissue samples in comparison with the control subjects was increased 3.2 fold ($p < .0001$). In contrast to CXCR4, the western blot analysis did not lead to any detection of protein bands for CXCL12, with the exception of the control protein (Fig. 2).

**Table 2.** Correlation between the expression of CXCR4 and CXCL12 at mRNA level and inflammatory markers analyzed within the AAA wall. Correlations were calculated using the Spearman rank correlation coefficient ($\rho$).

| | CXCR4 | CXCL12 | MSR1 | CD45 | CD3 |
|---|---|---|---|---|---|
| CXCR4 | | | | | |
| CXCL12 | 0.761** | | | | |
| MSR1 | 0.532** | 0.712** | | | |
| CD45 | 0.746** | 0.822** | 0.663** | | |
| CD3 | 0.535** | 0.549** | 0.414* | 0.581** | |

AAA = abdominal aortic aneurysm.
*$p < .05$; **$p < .01$.

### *Cellular localization of CXCR4 in AAA and controls determined by immunohistochemistry*

Histological and immunohistochemical analyses were performed for associating the expression of CXCR4 and CXCL12 to the different cell types within the AAA wall. The most abundant cells detected in AAA samples, as already shown in previous work,[4] were smooth muscle cells (SMCs) and inflammatory cells such as lymphocytes and macrophages. The immunohistochemical analyses of the chemokine receptor CXCR4 and its ligand CXCL12 are shown in Fig. 3. CXCR4 staining was positive for B and T lymphocytes, as well as for macrophages, indicating that these cells exhibited the highest expression of CXCR4. Interestingly,

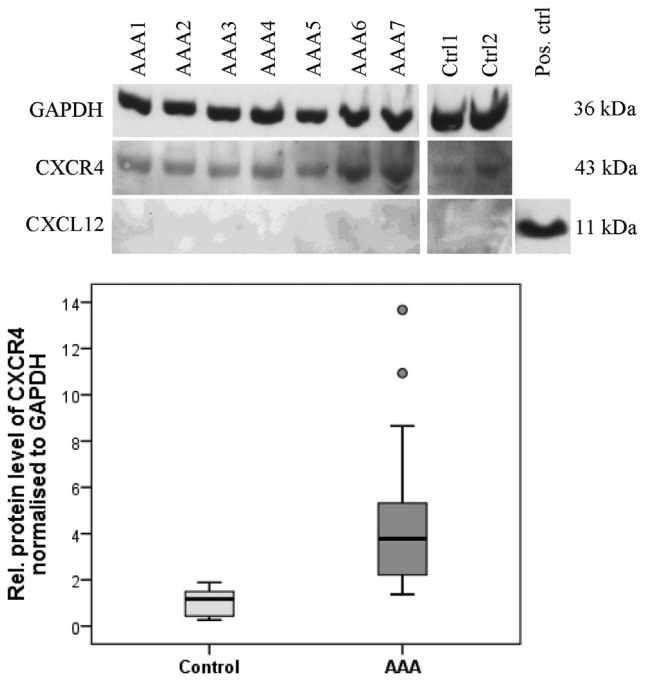


**Figure 2.** Expression of CXCR4 and CXCL12 at the protein level in AAA tissue samples ($n = 32$) compared with healthy control tissues (Ctrl, $n = 12$) analyzed by western blot. Recombinant CXCL12 protein was used as positive control (Pos. ctrl, 0.1 μg/lane). CXCL12 protein was not detected either in AAA or in healthy aorta. The intensities of the bands following blotting and chemiluminescence detection were standardized to GAPDH. AAA = abdominal aortic aneurysm; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. The Mann—Whitney U test was used.

neovessels were also partially positive for the staining (data not shown). With regard to CXCL12, immunohistochemical analysis only revealed positive staining for plasma cells (Fig. 4). All other cells in the AAA wall and in control aorta were negative for CXCL12.

## DISCUSSION

In this study it has been demonstrated that the expression of CXCR4 and its ligand CXCL12 is significantly upregulated in human AAA compared with non-aneurysmal aortic tissue. Furthermore, CXCR4 is mainly localized in inflammatory cells, in particular in B and T lymphocytes, as well as in macrophages.

AAA is a complex degenerative disease, characterized by the accumulation of infiltrating cells such as macrophages and lymphocytes.[6,28—37] The inflammatory processes within the AAA wall are initiated by multiple factors, including various pro-inflammatory cytokines, chemokines, and their receptors. A special focus has recently been devoted to the chemokine receptor CXCR4 and intensively investigated in various tumors, cancer,[21,22] and brain disorders,[23,24] as well as in atherosclerosis,[28] which is also known to be associated with AAA progression. CXCR4 has been shown to be critical for adhesion and/or migration by mediating trafficking and activation of lymphocytes, suggesting the involvement of CXCR4 in tumor progression, invasion, and metastasis.[19—23] Interestingly, only a few groups have so far analyzed the chemokine receptor CXCR4 and its ligand in human AAA in order to examine their role in lymphoid recruitment[25] and to evaluate the expression of CXCL12/CXCR4 in the aneurysmal aortic wall and during AAA progression in a mouse model.[26] In this study the focus was on the expression of CXCR4 and its ligand CXCL12 in the aortic wall of AAA patients and non-aneurysmal tissue subjects at the protein and mRNA levels and on the association of their expression in individual cells within the AAA wall.

First, to evaluate the presence of *CXCR4* and *CXCL12* in AAA tissue samples, the mRNA level was determined. The gene expression of *CXCR4* and *CXCL12* was detected in AAA samples as well as in non-aneurysmal aortas. Nevertheless, the expression of *CXCR4* showed a significant increase in AAA tissues samples of 9.6-fold, and 4.6-fold for *CXCL12*, compared with non-aneurysmal aorta. These results were in accordance with the previous published study from Lenk et al.,[29] who used Affymetrix and Illumina arrays on 10 AAA tissues samples and 10 control aortae. Lenk et al.[29] observed significantly increased expression of *CXCR4* in AAA attributed to leukocytes. Furthermore, another study analyzed 15 AAA and 15 control aortic tissue samples using a custom designed AAA chip and observed 38 differentially expressed genes between AAA and control samples. One of these genes was CXCR4 with 6.9-fold increase in AAA wall.[30] Both studies were carried out with control samples obtained from age, sex, and ethnicity matched individuals and all were from the infrarenal region of the aorta. Consequently, upregulation of CXCR4 expression in AAA versus healthy aorta was confirmed, even if the control group was not matched. It is however of note that a correlation analysis to figure out any relationship between age and expression of either *CXCR4* or *CXL12* in the tissue samples was performed. No correlation was observed. Therefore, in accordance with the other already published studies, it was assumed that age did not affect the comparison between the study groups. Because both factors are associated with the activation of inflammatory cells, their gene expression was correlated with the expression of the following inflammatory cells markers: *MSR1*, *CD45*, and *CD3*. Interestingly, significantly positive correlations were observed between the gene expression of *CXCR4*, *CXCL12*, and all these inflammatory markers (Table 2), indicating that gene expression of *CXCR4* and *CXCL12* is concomitant with inflammatory cells within the AAA wall. The results were in agreement with previous studies that showed that *CXCR4* and *CXCL12* are upregulated in human[25] and in mouse AAAs.[26] Remarkably, *CXCR4* transcription was also detected in the healthy aorta, although it was associated with *CXCR4* expression mainly in inflammatory cells. This discrepancy might be explained by using elderly individuals as controls, who had already slightly enlarged intima containing monocytes and by the presence of inflammatory cells in the adventitia.

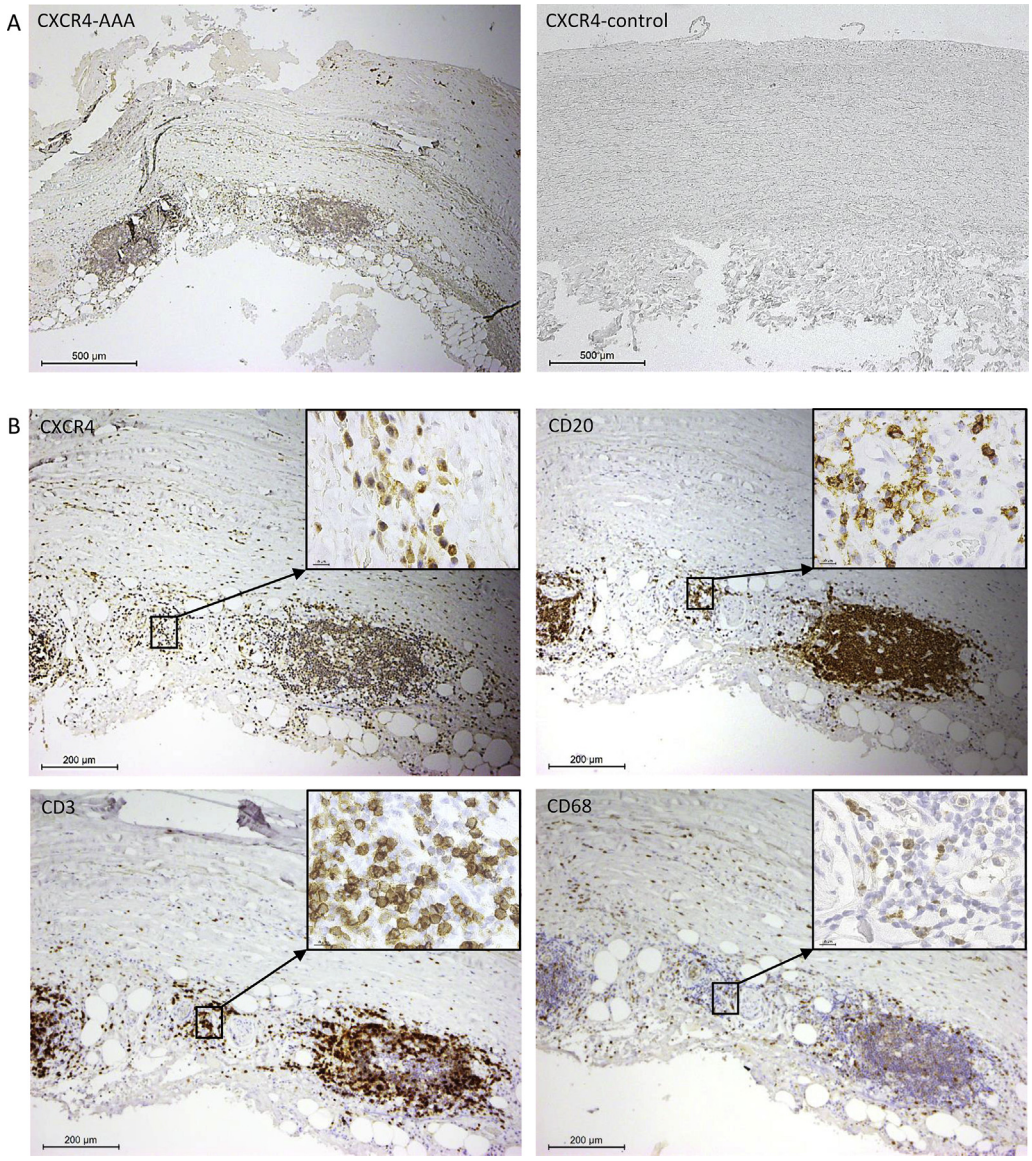


**Figure 3. (**A) Immunohistochemical analyses of CXCR4 in AAA tissue samples and in healthy tissues. (B) Selective immunohistochemical staining of CXCR4, CD68, CD3, and CD20 within the abdominal aortic aneurysm tissues samples. Positive cells are brown and counterstained with hematoxylin and eosin, showing cell nucleus in blue. Scale bars are 200 $\mu$m. The inserts are high power images of the selected regions. Scale bars are 10 $\mu$m.

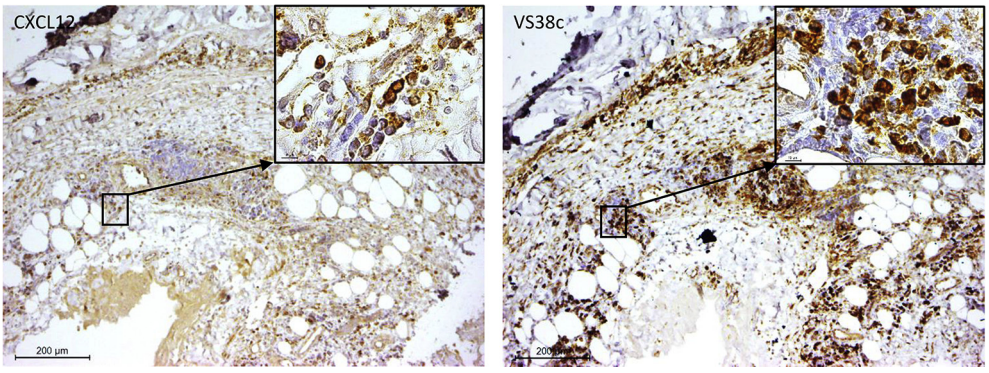


**Figure 4.** Immunohistochemical staining of CXCL12 and VS38c (plasma cells) within the AAA tissue samples. Positive cells are brown, the cells are counterstained with hematoxylin and eosin, showing the cell nucleus in blue. Scale bars are 200 $\mu$m. The inserts are high power images of the selected regions. Scale bars are 10 $\mu$m.

The results show an unambiguous increase in the expression of CXCR4 also at protein level. Interestingly, even if no expression of *CXCL12* at the mRNA level was found, no protein could be detected. These results are in discordance with the findings of Ocaña,[25] who detected CXCL12 in adventitia of the AAA wall. This apparent disagreement could be explained by differences in the methodology. Ocaña et al. analyzed the expression of CXCL12 by immunohistochemical staining[25]; in contrast, in this study evaluation was by expression via western blot, which is often less sensitive than immunohistochemistry. Using recombinant CXCL12 protein, the limit of detection was 0.1 μg. Consequently, if the protein concentration was lower, it could not be detected in the western blot analysis. Since the CXCL12 positive cells in the study of Ocaña et al. expressed neither CD45 marker of inflammatory cells nor smooth muscle actin or CD34, the precise source of CXCL12 could not be identified.[25] This work focused on inflammatory cells within the AAA, which was negative for CXCL12, as in the study of Ocaña et al.[25] However, CXCL12 positive cells associated only with plasma cells were detected. This can be interpreted such that these cells might be activated by an autoimmune response in the AAA wall as result of chronic inflammation.[38] Whether plasma cells are capable of expressing CXCL12 has to be further elucidated. Furthermore, the lack of detection of CXCL12 at the protein level could also be for the following reasons. First, protein and mRNA expression levels are known to be independent of each other.[39] Second, the half life of proteins is often different from that of the corresponding mRNA.[40] In accordance, CXCR4 as a transmembrane receptor is a stable protein. In contrast, the half-life of CXCL12 has been described as rather short.[41] Thus, the instability of CXCL12 might be another reason that this ligand could not be detected at the protein level.

Finally, to determine the cells within the AAA wall that express CXCR4 or CXCL12, consecutive immunohistological staining was performed for B and T lymphocytes and macrophages. These cells have already been classified as important sources of inflammatory processes, which invade the aortic vessel wall during the pathogenesis of AAA.[4,6] The results indicated that B and T lymphocytes as well as macrophages express CXCR4. Furthermore, similarly to CXCL12, CXCR4 was detected in plasma cells also. These cells differentiate from B cells and produce antibodies from the precursor B cell. The presence of plasma cells and B lymphocytes in the aneurysm,[4] together with the expression of CXCR4/CXCL12, further supports the assumption that an autoimmune reaction might be also involved in AAA disease progression. Additionally, CXCR4 and CXCL12 positive cells were not detected in healthy aorta tissues. These findings confirm once again the critical role of inflammation, which participates in a negative remodeling of extracellular matrix throughout the arterial wall leading to the vessel wall instability, AAA progression, and finally to rupture.[4,33–37] Furthermore, these results are in accordance with a recent study demonstrating that the CXCL12/CXCR4 pathway in the development and progression of AAAs is triggered through the accumulation of macrophages within the diseased aortic wall.[26] Moreover, Bot et al.[28] recently described that blockade of CXCR4/SDF-1α on leukocytes induces atherosclerotic plaque progression in mice. Taken together, these results emphasize that the expression and appearance of CXCR4 may correspond with the AAA progression and might be a potential target for detection of inflammatory activity in AAA patients.

### *Study limitation*

The non-aneurysmal control group used in this study was not age matched with the AAA study group, which could be a source of potential bias. It is extremely difficult to obtain healthy aortic samples, thus the available tissue material had to be used. It is also of note that no information was available with regard to medical history or medication of the healthy donors, such as hypertension, CKD, or diabetes mellitus, or the administration of statins, which could also influence the expression of the studied inflammatory markers. Furthermore, the immunohistochemistry results strongly depend upon the antibody used. Several antibodies were tested for both CXCR4 and CXCL12 to receive optimal staining outcome; nevertheless, especially with regard to CXCL12, the negative results might also be due to the insufficient sensitivity of the antibodies used in the study. Additionally, it is important to mention that the results provide only an association between the expression of CXCR4 and the inflammatory markers rather than the mechanism of biochemical cascade, which can be partially caused by global increase in gene expression in the inflammatory cells. Further functional studies are therefore necessary to elucidate the exact role of CXCR4/CXCL12 in AAA.

In summary, in contrast to other studies that analyzed CXCR4 separately either at mRNA level using array chips or by immunohistochemistry, this study focused on all three techniques: PCR, western blotting, and immunohistochemistry. The results demonstrate that CXCR4 is abundantly expressed in AAA tissue samples at both the RNA and the protein level compared with non-aneurysmal tissues. Furthermore, CXCR4 is particularly co-localized with inflammatory cells in the AAA wall. Thus, CXCR4 may serve as a potential target to detect non-invasively inflammatory activity in the diseased aortic wall. Further studies are necessary to validate the role of CXCR4 in the inflammatory proteolytic processes within the human AAA wall.

## CONFLICT OF INTEREST

None.

## FUNDING

None.

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